

Morphological Variations Among Larval-Postlarval Intermediates Produced by Eyestalk Ablation in the Snapping Shrimp *Alpheus heterochaelis* Say

PAUL S. GROSS^{1,*} AND ROBERT E. KNOWLTON²

¹*Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, South Carolina 29425; and* ²*Department of Biological Sciences, George Washington University, Washington, DC 20052*

Abstract. The eyestalk of many crustaceans contains the X-organ, the presumptive site of production and release of many protein and peptide hormones into the hemolymph. Removal of the eyestalk deprives the animal of these hormones and is known to affect many physiological processes in the adult and developing larva. In the snapping shrimp *Alpheus heterochaelis* Say, eyestalk ablation performed early in larval development has profound effects on morphogenesis, causing the appearance of supernumerary larval stages, accompanied by retardation and even complete arrest of morphogenesis. In this study, we examined the effects on morphogenesis of bilateral eyestalk removal at carefully controlled intervals. We found that the crucial point for this operation—the point at which the animal attains the ability to metamorphose fully—is just before the onset of ecdysis to the third instar. Additionally, the pattern of development and morphogenesis among body segments follows a discernible double gradient pattern along the anterior-posterior axis in which the extremities of the animal attain the potential for morphogenetic advance prior to the central thorax. This pattern of morphogenesis, punctuated by ecdysis, is a continuous rather than a stepwise or compartmentalized phenomenon.

Introduction

In decapod Crustacea, larval morphogenesis generally consists of small increments of structural change (from the initial hatching morphology) manifested as several zoeal

instars, culminating in a distinct change (metamorphosis) to a “postlarva” or “megalops” that more closely resembles the adult form (Williamson, 1982; Felder *et al.*, 1985). The metamorphic molt is usually of sufficient magnitude for the postlarva to assume the life habit of the adult, but some larval features may be retained until the next molt, to a juvenile form that is similar to the adult except for size.

Morphogenesis and molting history can be affected by environmental factors such as diet, temperature, and photoperiod (reviewed in Knowlton, 1974), and by hormonal cues. An “X-organ–sinus gland complex,” located in each of the paired eyestalks just medial to each optic complex, has long been known as a source of many hormones that can affect various physiological processes either directly or indirectly in adult decapods (reviewed in Carlisle and Knowles, 1959; Passano, 1960, 1961; Welsh, 1960; Kelly, 1967; Jenkin, 1970; Kleinholz, 1976; Cooke and Sullivan, 1982; and Fingerman, 1987). This complex also plays a role in control of larval development (reviewed in Christiansen, 1988; Charmantier and Charmantier, 1998). Eyestalk ablation has been shown to produce supernumerary larval stages, larval-postlarval intermediates, or both in various crustaceans such as *Rhithropanopeus harrisi* (Costlow, 1966) and *Homarus americanus* (Charmantier *et al.*, 1985; Charmantier and Aiken, 1987).

Larvae of the snapping shrimp *Alpheus heterochaelis* Say from coastal North Carolina (described by Knowlton, 1973) exhibit some unique developmental features. The larval phase is abbreviated, lasting only 4–5 days when reared at 22–25°C and consisting of three larval (zoeal) and one postlarval stage. The larvae hatch with a large amount of stored yolk and oil, which they apparently use as the sole source of energy throughout the larval period. Exogenous

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* To whom correspondence should be addressed. E-mail: grossp@musc.edu

feeding does not begin until the postlarval stage when the mouthparts develop into functional feeding organs (Gross and Knowlton, 1991).

Eyestalkless *A. heterochaelis* larvae have been observed to form larval-postlarval intermediates consistently as long as eyestalk removal is performed prior to the midpoint of larval Stage III (Knowlton, 1988, 1994). An array of different morphologies was discerned, depending on the time (during the second and third larval instars) that the eyestalk extirpation was performed. Knowlton (1994) described four types of larval intermediates, designated stages IVA through IVD, each possessing more developmentally advanced features and occurring between stage III and the postlarva. In his study, operations were performed once a day at the same time of day over the 4- to 5-day larval period. In the present study, this protocol was refined by increasing the number of time points at which operations were performed to seven (*i.e.*, at 2 h, at 20 h, and at subsequent 10-h intervals over the duration of larval development), utilizing more animals at each time point, and maintaining the animals over several instars (molts). Two series of experiments, each involving larvae hatched from eggs borne by four different females, were executed. Specific objectives of these experiments were (1) to ascertain whether morphogenesis occurs in discrete steps or is continuous and (2) to establish if extirpation at various times results in developmental arrest (either complete or delayed), and whether there is a critical threshold time after which development to the postlarval stage is determined.

Materials and Methods

Collection and maintenance of adults

Ovigerous adult female specimens of *Alpheus heterochaelis* were collected periodically during the summer months at three sites ("Duncan's Green," "Research Cove," and Radio Island) in Beaufort, North Carolina. Each shrimp was maintained individually in a 2-l aquarium containing 1.5 l of aerated (by pump and stone) seawater and an oyster shell "house" for the shrimp to hide under. The animals were maintained on a cycle of 14 h daylight and 10 h darkness and were fed TetraMin large flake fish food *ad libitum*. Gravid females were assessed daily for brood maturity. To establish the absolute age of any brood, only larvae for which the hatching event was actually observed were used.

Rearing of larvae

Seawater used in all experimental procedures was prepared as described in Gross and Knowlton (1997). Hatched zoea larvae were maintained in UV-treated seawater, which was further filter sterilized through a 0.45- μm filter to which the following chemicals were added: 50 ng/ml amphotericin

B, an antifungal agent, plus 50 $\mu\text{g/ml}$ each of the antibiotics streptomycin sulfate and ampicillin (ICN Biochemicals, Costa Mesa, CA). Larvae were maintained in 10 ml of processed seawater, changed daily, in an incubator at a constant 25°C to provide optimum development and a "standard" 4-day larval development pattern for control animals (Knowlton, 1973). When animals reached the postlarval and juvenile condition, they were fed freshly hatched *Artemia salina* (brine shrimp) nauplii *ad libitum*. General laboratory procedures are given in Gross and Knowlton (1997, 1999).

Protocol of operations

Immediately after all larvae in a particular brood had molted to larval stage II (about 2–3 h post-hatching), a subset of the brood, designated t_2 (for 2 h post-hatching) was subjected to eyestalk extirpation. A second population was ablated 18 h after t_2 and designated t_{20} . Eyestalk ablations were then performed at 10-h intervals after t_{20} up to 70 h, with groups being designated accordingly. In a previous study, it was found that larvae ablated at 10 h post-hatching (t_{10}) closely resembled those of the t_{20} group (data not shown; Gross and Knowlton, 1991). Typically, eyestalk ablations at t_2 and t_{20} occurred during Stage II and all other ablations during larval stage III, with t_{30} occurring almost immediately after the molt to stage III. A number of larvae in each brood were retained as unoperated controls. The control group and each group of larvae designated for surgery at a particular time point had roughly equal numbers of animals.

Analysis of intermediate morphologies

In the first series of experiments, a total of 274 larvae from four broods were used. After the final set of operations at t_{70} , each larva was individually monitored as it molted to the intermediate form (stage IV of Knowlton, 1994) or postlarva (in controls). Data pertaining to size differences are given in Gross and Knowlton (1999). At this time, the animal was preserved in 70% ethanol (EtOH). Each larva was then cleared in 5% KOH, stained using carmine-borax/35% EtOH, and placed in 100% glycerol by serial transfer, in preparation for structural examination and measurement (Guyer, 1906; Gross, 1995). Glycerin was chosen as the final medium because it maintains the clarity of the stained specimen; it is significantly more viscous than water, giving the specimen a greater internal rigidity; and it causes the otherwise brittle exoskeleton to become pliable, allowing the appendages to be moved and posed easily during analysis of larval characteristics. Both dissecting and compound microscopes were used to analyze morphological features of the fourth and fifth instar intermediate forms with respect to time of ablation, and were related to analogous morphological characters found in control postlarvae and juveniles.

In the second series of experiments, a separate set of 629 larvae was used. The procedure was similar to that already outlined except that the larvae were not sacrificed but were allowed to continue development through subsequent instars (molts). For consistency, operations on larvae were performed at the same time points. As they developed, live animals were examined for morphological diagnostic characteristics (along with instar duration and mortality; see Gross and Knowlton, 1997). Each larva was assigned a substage or "form" designation based on the system proposed by Knowlton (1994). Two behavioral characters were also recorded after each molting: (1) the orientation of the larva—dorsal side up (walking posture), consistent with an adult benthic life habit, or dorsal side down (swimming behavior), exhibited by pelagic larva; and (2) consumption of *Artemia salina* nauplii, as evidenced by food (opacity) in the stomach.

Results

Analysis of fourth instar (stage IV intermediate) morphology

The range of intermediates between stage III (third instar) and the postlarva (normally fourth instar) produced by bilateral eyestalk extirpation in these experiments is consistent with the four broad categories (*i.e.*, IVA-IVD) established by Knowlton (1994), but upon closer examination of individual specimen morphologies, some modifications of these broad categories are indicated. With more frequent ablation and monitoring, 10 forms were discerned, whose major structural features are summarized in Table 1.

Stage IVA, as described by Knowlton (1994), differs from stage III (see Knowlton, 1973) only with respect to abdominal development, and two distinct subtypes of the IVA form were discerned. In general, Stage IVA₁ is typified by very little morphological change. Stage IVA₁ animals were "more postlarval" with respect to the tail region (*e.g.*, formation of uropodal endopodite and acquisition of the postlarval number of terminal setae on the telson). However, the telson of each IVA₁ larva retained its stage III shape, with the uropodal exopodite being underdeveloped, lacking both the spine located on the outer margin and the distal-most joint, and the endopodite being smaller than that of a normal postlarva in most cases (69.6% of those examined). Stage IVA₂ animals often possessed pleopods about normal postlarval size and bearing long plumose setae (as in the postlarva); less often (in 31.6% of those examined), the uropodal endopodite was reduced. The IVA₁ form was encountered exclusively among larvae ablated at t₂, whereas the IVA₂ form was encountered primarily among t₂₀ larvae (Table 2).

Stage IVB, as described by Knowlton (1994), incorporates the abdominal development seen in the IVA₂ form with an array of morphogenetic advances in the head region,

Table 1

Recognition characters of expanded intermediate morphogenetic categories (forms)

Form	Additional key features
A ₁	Pleopods aetose (or with very short setae)
A ₂	Pleopods setose (=Knowlton's stage IVA)
A/B	Antennal flagellum with 5 segments Antennule flagella as in stage IVA
B	Antennal flagellum with 9–16 segments, protopod with basicerite Antennular inner and outer flagellum segmented, peduncle with stylocerite (=Knowlton's stage IVB)
B/C	Exopods of pereopods 1–4 setose Exopod of pereopod 4 shortened
C ₁	Telson lacks pair of dorsal spines
C ₂	Exopods of pereopods 1–4 shorter but setose Endopods of pereopods 1–5 with more claw-like dactyls (=Knowlton's stage IVC)
C/D	Exopods of pereopods 1–3 setose and shortened Exopod of pereopod 4 aetose, nearly vestigial
D	Exopods of pereopods 1–2 shorter but setose Exopods of pereopods 3–4 aetose, nearly vestigial (=Knowlton's stage IVD)
D/PL	Exopod of pereopod 1 setose, nearly vestigial Exopods of pereopods 2–4 aetose, vestigial
E	Exopods of pereopods 1–4 aetose, vestigial (as in postlarva) Telson with two pairs of spines on dorsal surface (as in juvenile)
F	Exopods of pereopods 1–3 vestigial (absent on 4) Telson with two pairs of spines on dorsal surface (as in juvenile)
G	Exopods of pereopods 1–2 vestigial (absent on 3 and 4) Telson with two pairs of spines on dorsal surface (as in juvenile)

but development in the thoracic region is not yet seen. Much variation in form was noted among IVB larvae, especially with regard to the development of head appendages. A few larvae had the full (*i.e.*, normal postlarval) complement of 4 segments on the outer flagellum of the antennule, but typically only 1–2 segments were present. Conversely, the inner flagellum of the antennule usually was postlarval in form (possessing 3 segments), but a few larvae had 1–2 segments added. A wide range of antennal flagellum segments (1–22) was displayed by animals in this form, but even when a full complement of segments was present (22–26), the overall length of the antennal flagellum was significantly different ($F = 77.30$, $P < 0.05$) than in the normal postlarva. Although the third and final segment of the antennular peduncle was usually present, the basicerite was rarely added and the statocyst never found among stage IVB animals. This form was most often produced when eyestalks were removed late in stage II (t₂₀) and early in stage III (t₃₀), but many other intermediate forms were also produced as a result of eyestalk ablation during this period (Table 2). Some larvae, primarily in the t₂₀ group IVA/B, showed very little advance over the IVA₂ form but did have 1–2 segments added to the antennal flagellum (Table 1). The A/B designation indicates that these larvae were not quite equivalent to either the IVA₂ or IVB forms.

Table 2

Distribution of fourth instar forms at each operative time point (percent of total survivors per operative time point)

Fourth instar form	Operation time (in hours)							Control
	t ₂	t ₂₀	t ₃₀	t ₄₀	t ₅₀	t ₆₀	t ₇₀	
A ₁	83.7 (72)							
A ₂	12.8 (11)	40.3 (27)						
A/B	1.2 (1)	20.9 (14)	2.5 (2)					
B	2.3 (2)	37.3 (25)	38.0 (30)	9.9 (8)				
B/C			2.5 (2)					
C ₁			13.9 (11)	4.9 (4)				
C ₂		1.5 (1)	22.8 (18)	14.8 (12)				
C/D			3.8 (3)	1.2 (1)				
D			12.7 (10)	38.3 (31)	10.6 (9)	3.5 (3)		
D/PL				1.2 (1)	4.7 (4)	2.3 (2)	2.3 (2)	
PL			3.8 (3)	29.6 (24)	84.7 (72)	91.2 (81)	97.7 (85)	100 (151)

Data in parentheses are actual number of animals (*n*) assigned to the category. Designations A₁ to D/PL indicate fourth instar forms that are intermediate between larva and postlarva (PL).

In Knowlton's (1994) stage IVC, the head and abdominal regions are morphologically postlarval, but the thoracic appendages are only partially developed toward the postlarval form. Like the previous intermediate forms, the IVC form generally swims ventral side up and is incapable of feeding. In the present study, three additional IVC types, based on the degree of morphological advance in the thorax and abdomen, were distinguished (Table 1).

Stage IVB/C larvae, found only among animals ablated at t₃₀ (Table 2), exhibited most of the characteristics of Knowlton's stage IVC, but only the exopod of pereopod 4 showed reduction in size and loss of swimming function. Animals designated stage IVC₁ lacked the pair of spines on the dorsal side of the telson about three-quarters of the length distal from the joint connecting the telson to the sixth abdominal somite (as in the postlarva), whereas those designated stage IVC₂ possessed these spines. In addition, the IVC₁ form was more commonly found in animals from the t₃₀ group (relative to the t₄₀ group). The IVC₂ form was more common than the IVC₁ form among larvae ablated at t₄₀ (Table 2).

Some characteristics were shared by all larvae classified as stage IVC. For example, although the uropodal exopodite is jointed in the postlarva, it was never jointed in animals classified as stage IVC; however, the typical postlarval spine located on the outer margin was always acquired. The antennal flagellum was essentially postlarval in its length and number of segments. In some animals, the inner and outer antennular flagella had 3 segments instead of the postlarval 4. The antennular peduncle was always 3-segmented and the basicerite was usually present, but the statocyst was never completely formed.

The thoracic appendages of the stage IVC form (both IVC₁ and IVC₂) were in many ways still similar to those of

earlier intermediate forms, and morphogenetic advance was more pronounced in posterior thoracic appendages than in the anterior ones. The exopods of pereopods 1 and 2 exhibited almost no change relative to stage III. However, those of pereopods 3 and 4 were dramatically shortened (but not to the extent seen in the normal postlarva) and curled in appearance, with 4–6 misshapen setae on each terminus. Pereopod 5, which lacks a swimming exopod throughout development, remained 7-segmented, but its dactyl was blunter than the very long styliiform appendage associated with Stage III. All joints on this appendage appeared to be functional, with the joint between the merus and carpus modified to form a knee-like joint more suited to walking. The endopods of pereopods 3 and 4 underwent proportionately more morphological change (compared with those of pereopods 1 and 2). In most animals, they had the full complement of 7 segments (sometimes 6), with the styliiform dactyl being replaced by a blunter form; joints on these appendages were always functional. The endopod of pereopod 2 displayed the most segments (8–10), but development was incomplete as the joints were not articulating. New segments were usually added to the endopod of pereopod 1, but the joints of this appendage were never fully articulated at this stage.

Individuals assessed as stage IVD (Knowlton, 1994), as exemplified by larvae ablated at t₃₀–t₆₀ (Table 2), possessed most of the characteristics of the postlarva while still retaining a few larval characteristics. In these animals, the telson was postlarval in shape, possessing the typical 4 + 4 terminal setation pattern and the characteristic pair of spines located on the dorsal surface. The uropods were fully developed, with the exopodite bearing both the outer spine and distal joint. The antennal flagellum was as long as that of the typical postlarva, with a full complement of segments (22–

26). Some specimens possessed 3-segmented inner and outer antennular flagella (rather than the usual 4 segments), but the peduncle was always 3-segmented, with basicerite and statocyst fully developed. These larvae exhibited complete transformation of pereopods 3–5 to postlarval form. The endopod of pereopod 1 was morphologically postlarval in this stage as well, always being composed of the full complement of 6 segments, with the elbow-like joint between merus and carpus, and completion of the joint between the propodus and dactyl to form a functional chela. The endopod of pereopod 2 still showed some variability in segment number (9–10), but was functionally complete, with an articulated chela and an elbow-like joint between the merus and first carpal article. Exopods of pereopods 3 and 4 were usually devoid of setation and nub-like (Table 1), as in the postlarva; in some specimens, however, these exopods were longer than that of a normal postlarva. Exopods of pereopods 1 and 2 were not functional but had not attained the naked nub-like appearance of the postlarva. Stage IVD animals typically walked ventral side down, like the postlarva, consistent with the presence of a fully developed organ of balance (statocyst). They were usually capable of feeding (also like the postlarva), as evidenced by the presence of *Artemia salina* nauplii remains in the stomach.

A few animals (C/D in Table 2) ablated at t_{30} and t_{40} possessed setose exopods on pereopods 1–3. Similarly, a significant proportion of those larvae ablated at t_{50} – t_{70} possessed setation only on the exopod of pereopod 1 (D/PL). These larvae appeared to be similar to the postlarva in other respects, except that the second pereopodal endopod occasionally had 9 segments rather than 10.

In summary, detailed examination of ablated larvae from each time point yielded in essence a continuum of stage IV morphologies between larval stage III and the normal postlarva, and larvae ablated at t_{30} and t_{40} showed the greatest variability in intermediate form morphology (Table 2).

Analysis of fifth instar morphology

Intermediate morphologies encountered at the fifth instar (*i.e.*, after four molts post-hatching), which is normally the molt to a juvenile form, are enumerated in Table 3 with the same nomenclature used to describe fourth instar forms. A stage V designation identifies fifth instar intermediates, which are still morphologically intermediate between larval stage III and postlarva, while E, F, and G designations are reserved for individuals with characteristics intermediate between the postlarva (PL) and juvenile (J).

At the fifth instar, some larvae continued to exhibit a lag in morphological advancement. Some larvae ablated at t_2 (IVA₁ in Table 2) did not exhibit any structural change after the molt to the fifth instar (Table 3) and were designated

stage VA₁ since another molt had occurred. However, most (55.5%) IVA₁ larvae exhibited slight morphological advancement (*i.e.*, they were morphologically identical to stage IVA₂) and were designated stage VA₂. A significant proportion of animals (39.7%) advanced to either stage VA/B or VB (equivalent to IVA/B and IVB, respectively). All but one of the t_2 animals died during this instar, with only a single stage VB larva surviving to molt unchanged to a sixth instar. A similar trend of developmental arrest is seen among larvae ablated at t_{20} : 75.8% of animals exhibiting stage IVA₂, IVA/B, or IVB morphology at the fourth instar (Table 2) did not advance morphologically beyond stage VB at the fifth instar (Table 3). However, three larvae from the t_{20} group molted to postlarval form, surviving to molt to a morphologically normal juvenile by the sixth instar. It is interesting that three t_{20} larvae had morphological features spanning the gap between form D and the juvenile. These three larvae (designated VD/PL/J) possessed setose exopods on pereopods 1 and 2 (as in form D) and had a telson morphology (*i.e.*, two pairs of dorsal spines) consistent with that of a juvenile.

Many (45.0%) of the larvae ablated at t_{30} , which as fourth instars formed a wide array of larval-postlarval intermediate forms (Table 2), molted to the juvenile form at the next molt; nine animals (15.0%) resembled normal postlarvae at the fifth instar (Table 3). A significant number of animals from this group exhibited characteristics, noted under E in Table 1, that were intermediate between the normal postlarva and juvenile. Some larvae ablated at t_{30} were found to possess the juvenile telson morphology with vestigial exopod nubs on pereopods 1–3, or on 1–2 only (remaining pereopods being uniramous); these intermediate forms were designated F and G, respectively (Table 3). Of the broad spectrum of intermediate stage IV types noted among larvae ablated at t_{40} (Table 2), the majority (70.5%) molted to juvenile at the fifth instar, but most of the others (E in Table 3) retained all pereopod exopod rudiments on pereopods 1–4. All larvae from the t_{50} – t_{70} groups molted to the juvenile form at the fifth instar.

All animals surviving to the sixth instar, which attained some semblance of postlarval morphology at the fifth instar, were found to exhibit morphology consistent with classification as juveniles. Comparing the distribution of larval intermediates found at the fifth (*vs.* fourth) instar, larvae ablated prior to the molt to stage III (t_2 and t_{20}) appeared, for the most part, to be incapable of completing development (metamorphosis). Conversely, those ablated after the molt to stage III (t_{30} through t_{70}) completed metamorphosis by the sixth instar. However, animals ablated at t_{30} (and to some extent at t_{40}) showed a definite lag in morphogenesis relative to larvae ablated at later times and were more prone to appear as postlarval-juvenile intermediates during the fifth instar.

Table 3

Distribution of fifth instar forms at each operative time point (percent of total survivors per operative time point)

Fifth instar form	Operation time (in hours)							Control
	t ₂	t ₂₀	t ₃₀	t ₄₀	t ₅₀	t ₆₀	t ₇₀	
A ₁	4.8 (3)							
A ₂	55.5 (35)	18.2 (6)						
A/B	11.1 (7)	9.1 (3)						
B	28.6 (18)	48.5 (16)	3.3 (2)					
B/C		6.1 (2)	1.7 (1)					
C								
D								
D/PL/J		9.1 (3)						
PL		9.1 (3)	15.0 (9)					
E			25.0 (15)	27.9 (17)				
F			3.3 (2)					
G			6.7 (4)	1.6 (1)				
J			45.0 (27)	70.5 (43)	100 (63)	100 (63)	100 (53)	100 (127)

Data in parentheses are actual number of animals (*n*) assigned to the category. Designations A₁ to D/PL/J indicate fifth instar forms that are intermediate between larva and postlarva (PL), designations E-G indicate intermediate forms between postlarva and juvenile (J)

Discussion

Distribution of larval intermediate types in relation to time of ablation

Results of our study indicate that in *Alpheus heterochaelis*, time of eyestalk ablation governs the amount of morphogenetic advance, such that more advanced forms are produced from successively later ablations (Table 2–3). Further, the array of larval-postlarval intermediate forms caused by eyestalk extirpation is virtually continuous. These forms (Table 1) resulted from operations carried out at seven different time points (t_x), with much morphological variation being noted within each t_x group as well as among groups. The range of morphogenetic variability was least among animals ablated either very early or very late and was at a maximum among the t₃₀ and t₄₀ groups. Larvae ablated at the earliest time point (t₂) were morphogenetically least advanced, most animals exhibiting the IVA₁ form (Table 2). This intermediate type was found only among larvae ablated at t₂ and not among larvae ablated 10 h post-hatching (Gross and Knowlton, 1991). The t₂₀ group included animals ranging in form from IVA₂ to IVB, but the IVA₂ form was never found among t₃₀ larvae, whose individuals exhibited IVB-IVD forms (Table 2). Among t₄₀ animals, occurrences of the IVB form were fewer, whereas intermediates exhibiting the IVD morphology were more common. Stage IVC (*i.e.*, IVC₁, IVC₂, or IVC/D) animals were distributed between t₃₀ and t₄₀ and were essentially restricted to these groups. Larvae ablated after t₄₀ exhibited progressively fewer intermediate types, and incidences of complete transformation to the postlarval form became the norm.

Crucial points in morphogenesis

The crucial point at which the *A. heterochaelis* larva becomes capable of completing morphogenesis to the juvenile condition (normally the fifth instar) seems to occur around the time of ecdysis to stage III. None of the larvae in the t₂ group changed structurally beyond form B (Table 2), and most of the t₂₀ animals died without morphological advance beyond form B/C (Table 3). A small number of animals from the t₂₀ group did succeed in attaining a postlarval morphology, or a form close to it (VD/PL/J), at the fifth instar, but only two individuals survived to molt to a morphogenetically normal juvenile at the sixth instar. Thus, with few exceptions, larvae ablated before the molt to stage III were incapable of morphogenetic advance to the postlarval or juvenile form. Similarly, in *Homarus americanus* (which exhibits a pattern of larval development similar to *A. heterochaelis*), Charmantier and Aiken (1987) noted that the critical period for eyestalk removal leading to developmental arrest and the appearance of larval-postlarval (stage IVa) intermediates was in molting stage D₁ of larval stage II. Conversely, *A. heterochaelis* larvae that underwent eyestalk ablation during stage III could complete morphogenesis to a feeding juvenile or could attain an intermediate morphology between postlarva and juvenile (*i.e.*, forms E, F, and G) during the fifth instar. However, a few animals ablated close to the completion of ecdysis to stage III did not advance beyond form B. Thus, the "turning point" separating completion of metamorphosis from developmental arrest seems to be at the interface between stages II and III at or about the time of ecdysis, which is in agreement with the findings of Knowlton (1994).

Whereas Knowlton (1994) did not note any further mor-

phological changes among larvae ablated during stage II (before attaining the IVA or IVB form), our more detailed analysis showed that by the fifth instar most larvae exhibited some change in morphology relative to their stage IV counterparts. The majority of t_2 animals advanced from form A₁ to the A₂, A/B, or B form, and most t_{20} animals surviving to a fifth instar also advanced slightly in form. Thus it appears that even though metamorphosis is blocked by ablation before the third instar, morphogenesis is not completely arrested. Even among larvae that can complete metamorphosis to the juvenile form, the effects of eyestalk ablation may only be overcome over multiple instars and elongation of the larval period (Gross and Knowlton, 1999). On the other hand, even though t_{30} and t_{40} larvae were able to complete metamorphosis to a morphologically normal juvenile by the sixth instar, there was a definite lag in morphogenesis.

Form D animals are substantially different and more advanced than those of form C since they possess fully functional (articulating) thoracic endopods (as in the postlarva) and are able to feed, orient themselves ventral side down, and walk. It is apparent that at about 35–40 h post-hatching the third instar larva attains the ability to make, after molting to the fourth instar, a more “complete” morphological conversion from a planktonic larval existence to a more adult benthic existence. Ablation after about 35 h post-hatching more frequently resulted in the stage IVD form or more advanced forms (up to and including normal postlarva—Table 2). Most t_{50} individuals exhibited morphology consistent with the postlarva (at the fourth instar). But morphogenesis was not “finalized” until late in the third instar, since larvae with form D features were seen even among t_{70} animals.

The nature of the morphogenetic continuum

Carlisle and Knowles (1959) postulated that factors located in the X-organ and released at the sinus gland control molting and that morphogenesis is a stepwise process which coincides with the molt cycle and may even be controlled by it. This does not appear to be the case for *A. heterochaelis* larvae, which, when their eyestalks are ablated at various times, display a continuum of intermediate morphologies independently of the molting cycle. If molting and morphogenesis were inextricably tied, then it would be reasonable to assume that premature molting would either fail to produce any morphogenetic advance or would yield a stepwise array of developmental intermediates. Knowlton (1994) observed a limited array of intermediates in experiments in which eyestalks were ablated only once per day. However, by increasing the number of time points for eyestalk ablation, a more continuous array of intermediates was produced that encompassed many more intermediate morphological forms. Our results argue against developmental patterns in higher Crustacea being “fixed” (Gurney, 1939), with all

individuals showing exactly the same changes at each molt; or “compartmentalized” (Fraser, 1936), with individuals exhibiting morphological variation within a limited range (compartment). Indeed, they provide ample evidence that development in these crustaceans is continuous—a gradual process in which different body segments attain the potential for differentiation into their adult morphology at different times. Removal of the eyestalk at intervals makes this aspect of gradual morphogenetic change more apparent and shows that molting and morphogenesis are concurrent but independently controlled processes.

The axis of morphogenetic change

Eyestalk ablation at various times interrupts the normal process of morphogenesis at different points along the morphogenetic continuum (described above). Differentiation appears to take place at different rates in each segment in a “double gradient” pattern. This is well evidenced by the descriptive data collected from groups of animals at each ablation time point. The ability to differentiate at the next molt is attained in the tail region first, with uropodal endopods and pleopods gaining the ability to differentiate to postlarval form (and size) as early as 2 and 20 h post-hatching, respectively. Under eyestalk control, ability to differentiate is attained in the head region slightly later, since larvae ablated at t_{20} and t_{30} begin to exhibit morphogenetic advances (changes in form and size of appendages) in the head region at the fourth instar. The segment bearing pereopod 5 gains the ability to metamorphose (*i.e.*, convert the styliform dactyl to a blunter form and shorten in overall length) at the fourth instar at about the same time as the head appendages (around t_{30}), reinforcing the idea that determination is advancing forward from the tail. Among eyestalkless animals, determination in the middle region (thorax) occurs later than in the head and tail region and is attained in exopods of the third and fourth pereopods well before those of the first and second.

Differentiation in form (*e.g.*, adding segments to appendages) precedes the potential for functional changes (*e.g.*, formation of movable joints). Pereopod 5 differentiates to postlarval length and segment number with the t_{30} group, but functional joints are not commonly found on this appendage at the fourth instar in these animals, whereas in the t_{40} group the fifth pereopod is usually fully functional by the fourth instar. Among larvae ablated at t_{30} , the third and fourth pereopodal endopods were equivalent to controls in length and segment number, but functional joints on these appendages were generally not seen until later (*e.g.*, among t_{40} or t_{50} animals). At the fourth instar, endopods of pereopods 1 and 2 both become functional among larvae ablated around t_{40} (or t_{50}). They lag behind pereopods 3–5 in onset of morphogenesis (reaching postlarval length and segment number) and attainment of functionality (articulating joints).

One can hypothesize that, under normal circumstances, the ability to metamorphose (at the molt to the fourth instar) is attained at different times by different body regions and even by specific segments within a body region of the *A. heterochaelis* larva. Factors in the eyestalk either control this process directly, or different body segments become receptive at different times to a factor or factors released by the eyestalk. Morphogenetic advance is not manifested until ecdysis, but the potential for morphogenesis toward the postlarval form starts early in the second instar, increasing gradually and continuously over the larval period. This potential for morphogenesis (differentiation at the next molt) is not the same along the posterior-anterior axis but instead may be visualized as a "double gradient" extending from the posterior and anterior ends toward the thorax. The trend is that the capacity to differentiate develops in the abdomen and abdominal appendages (uropods and pleopods) first, followed shortly thereafter by the head and its appendages, and lastly by the thorax. In the thorax, determination occurs earliest in the segments bearing pereopod 5 and maxilliped 1, while the last segment to be determined bears the second pereopod. Thus, the double gradient simultaneously extends forward from the fifth pereopod and backward from the first maxilliped, converging on the second pereopod.

Possible mechanisms of hormonal control

Several models could account for the production, through bilateral eyestalk ablation, of larval-postlarval intermediate forms and for the double gradient pattern of gradual determination. One explanation is that eyestalk removal deprives the developing larva of a critical regulatory principle or hormone that causes a given segment to transform from larval to postlarval morphology, and that this potential is realized gradually or at different times in different segments. The functional details of these mechanisms are still a matter of conjecture.

The simplest explanation for the appearance of larval-postlarval intermediate forms, advanced by Knowlton (1994), posits the existence of a morphogenetic hormone (MH) that is secreted by the eyestalk (presumably by the X-organ-sinus gland complex). This release would necessarily be gradual and begin early in stage II. Imam (1982) noted that certain tissues having a glandular appearance early in stage III differentiate in the larval eyestalk, only to degenerate in the postlarval eyestalk, surmising that these structures play a secretory role in the control of morphogenesis. A morphogenetic hormone would have to activate different segments at different times: either a segment could respond when the hormone reached a specific (and variable from segment to segment) concentration threshold in that segment, or it could respond only at a specific time in the morphogenetic continuum. If morphogenetic activation of

certain segments does depend on a certain MH threshold, then removal of the eyestalk (which deprives the developing larva of any further production of this hormone) should produce morphogenetic arrest in those segments—presumably the more central thoracic ones—that were below threshold at the time of eyestalk removal. This condition should be permanent for those segments below threshold, since circulating titers of the morphogenetic factor would be below threshold level at the time of ablation. Clearly this is not the case since almost all larval-postlarval intermediates produced by means of bilateral eyestalk ablation during stage III are capable of metamorphosis to a normal form, given enough time.

If specific segments became responsive to MH at different times during the morphogenetic continuum, then in order for morphogenesis to continue in those segments that are activated later or at a slower rate, MH would have to be available in the circulation well after its source was removed. MH would thus have to be an extraordinarily long-lived factor, which is not typical for circulating hormones. Alternatively, MH may be released in small amounts during stage II (enough to instigate abdominal morphogenesis even after eyestalk ablation), but this release is either not adequate to activate other body regions or other body regions are unresponsive at this stage. After the molt to stage III, MH may be released in a quantity high enough to instigate morphogenesis in all body regions but in a quantity too low to control the timing of morphogenesis (since larvae ablated at t_{30} exhibit a wide variety of larval-postlarval intermediates but can eventually attain the normal juvenile form), especially in the thoracic region. That is, circulating titers of MH may control the timing of morphogenesis in each segment, but activation depends on a certain basal concentration of MH being released.

In insects, morphogenesis is not controlled by a hormone that instigates the process but by a metamorphosis-inhibiting factor (MIF) or juvenile hormone (JH) that inhibits its onset until a specific time (pupation). This hormone (JH) is maintained in high titers during the larval phase and drops precipitously at metamorphosis. If the control of metamorphosis were similar in the Crustacea, it seems more likely that the eyestalk produces some sort of regulatory factor, which controls morphogenesis through a second hormone.

Some inferential evidence for a crustacean juvenile hormone or metamorphosis-inhibiting factor does exist. Freeman and Costlow (1983) found that whole-body extracts of stage III *Rhithropanopeus harrisi* larvae inhibited resorption of the large dorsal spine (characteristic of the zoea) *in vitro*. Further, Laufer and Borst (1988) have advanced the idea that methyl farnesoate (MF), produced in the mandibular organ (MO), functions as a MIF; they established that control of its secretion is under the influence of tissues in the eyestalk. It has been shown conclusively that the X-organ-sinus gland complex secretes a mandibular organ inhibiting

hormone (MOIH), which controls titers of MF by inhibiting its production within the MO (Liu and Laufer, 1996; Wainwright *et al.*, 1996; Liu *et al.*, 1997)

The results of the present research cannot conclusively distinguish between Knowlton's "MH hypothesis" and the existence of a MIF, and it may be that both systems exist in tandem. Knowlton (1994) asserted that in such a case it would be a balance between MIF and MH that controls the onset and timing of morphogenesis. But on the basis of this and other research (*e.g.*, Freeman and Costlow, 1983), it is possible that one or more factors, released from the eyestalk, inhibit MIF function or release from a second gland, and that different body segments have unique thresholds to MIF titers. The hypothesis that the eyestalk produces a regulatory hormone modulating the release of MIF would partially explain the double gradient pattern of morphogenetic potentiation in *A. heterochaelis* larvae if, as postulated by Borst *et al.* (1987), the mandibular organ is the source of MIF and that MIF is in fact MF. Simple proximity to this gland could be related to circulating titers of MIF. The extreme ends of the animal could be subjected to lower amounts of MIF naturally, by virtue of being the most distant from the mandibular organ, and thus would be released from inhibition earlier, while the central body segments would be proportionately more affected by MIF and released from inhibition later as titers of MIF dropped off under the influence of eyestalk secretion. Release of MIF and circulating titers may be controlled by a factor released from the X-organ–sinus gland complex in the intact animal. In eyestalkless animals, responsiveness to MIF may drop over time (even though circulating titers may not), or circulating titers of MIF may fall even without the influence of the factor or factors released by the eyestalk (though more gradually than in intact animals). This could produce the double gradient effect as each segment becomes progressively less responsive or titers fall below the unique threshold of each segment sequentially.

The idea that MF is the active JH in crustaceans is still very much in doubt, but some compelling evidence does exist. Exposure to MF has been shown to retard metamorphosis in *H. americanus* (Borst *et al.*, 1987), to retard early development in *Macrobrachium rosenbergii* (Abdu *et al.*, 1998a), and to have a juvenilizing effect on *Balanus amphitrite* larvae (Smith *et al.*, 2000). However, as pointed out by Abdu *et al.* (1998b), these effects could be due to nonspecific toxicity related to continuous exposure to MF. These same authors (Abdu *et al.*, 1998b) present a strong case that MF is the direct hormonal agent controlling metamorphosis; the arguments are particularly convincing when coupled with the identification of an eyestalk-related MOIH. In relation to these studies, the present work clearly indicates that MIF, be it MF or some other factor, controls metamorphosis directly and that the relationship between morphogenesis and molting is not one that affects molt

timing but one that affects hormonal activation of the remodeling process itself.

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